

# CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

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# Development of porcine respiratory and reproductive syndrome virus replicon vector for foot-and-mouth disease vaccine

**Purpose:** Foot-and-mouth disease (FMD) is an economically important global animal disease. To control FMD virus (FMDV) outbreaks, a lot of different novel approaches have been attempted. In this study, we proposed a novel porcine reproductive and respiratory syndrome virus (PRRSV) as a replicon vector to express FMDV structural protein.

**Materials and Methods:** PRRSV infectious clone (PRRSVK418DM) was used to develop an expression vector through the reverse genetic manipulation of PRRSV; FMDVP12A3C gene of serotype O was synthesized and used for an antigen. MARC-145 cells (African green monkey kidney epithelial cell line) were used for electroporation mediated transfection. The transfection or the expression of P12A3C and N protein of PRRSV was analyzed by either replicon containing PRRSV alone or by co-infection of helper PRRSV.

**Results:** We constructed PRRSVK418DM replicon vector containing FMDVP12A3C, and genome sequences were confirmed by subsequent sequence analysis. *In vitro* expression of P12A3C and PRRSV N protein was confirmed by immunofluorescence antibody assay using antibodies specific for PRRSV N protein (anti-PRRSV N MAb), FMDV-VP1 (anti-VP1 MAb).

**Conclusion:** The results indicate that PRRSV replicon vector can be a promising novel vector system to control FMDV and useful for vaccine development in the future.

**Keywords:** Replicon, FMDV, PRRSV, Genetic vectors

## Introduction

Foot-and-mouth disease virus (FMDV) is an important animal pathogen that infects cloven-hoofed animals, and economically devastating disease of livestock worldwide [1]. The virus causes fever, lameness blisters on the feet and mouth, and the appearance of vesicular lesions in the mouth, tongue, nose, feet and teats [2]. FMDV rapidly replicates in the pharynx of hosts, invades the blood stream within 48 hours, thereafter lesions appear in the mouth and feet and spreads via aerosol, either by direct contact, or by contaminated animal products [3,4]. However, a recent report suggests that pigs are the significant factor in the spread of the disease since a single pig releases as much aerosol virus as 3,000 cattle in a short period of time [5].

FMDV is a non-enveloped virus with an icosahedral capsid belonging to the *Aphthovirus* genus of the *Picornaviridae* family. The genome is a compact positive-strand RNA about 8,300 nucleotides long with a single open reading frame (ORF) [6]. The ge-

nome is translated as a single ORF into a precursor polyprotein and the precursor protein is cleaved by viral coded proteases into both the intermediate and mature structural and nonstructural (NS) viral proteins. Based on the initial cleavage products, the genome ORF is divided into four regions including the L, P1, P2, and P3 region, respectively [7]. The P1 region of the genome is encoding four viral structural proteins (VP4, VP2, VP3, and VP1). Following the P1 region is the P2, encodes three viral NS proteins (2A, 2B, and 2C), and the P3 region, encodes NS proteins 3A, three copies of VPg (3B1, 3B2, and 3B3), 3C protease ( $3C^{pro}$ ), and 3D polymerase ( $3D^{pol}$ ). The protease 3C plays crucial role in the cleavage of viral structural proteins and enables the proper folding and assembly of the FMDV capsid in the infected cells [7-9].

FMDV is one of the highly antigenic variable viruses, as a result of error-prone replication, and the lack of  $3D^{pol}$  gene proofreading and postreplicative repair activities. Therefore, the FMDV consists of the seven serotypes including type O, A, C, SAT-1, SAT-2, SAT-3, and Asia-1; 64 subtypes. Among the seven serotypes of FMDV, the serotype "O" is the most common and it is prevalent in China and its surrounding countries. Furthermore, serotype O has been detected in South Korea, during the massive outbreaks of foot-and-mouth disease (FMD) in 2011 [10].

The development of FMDV vaccine is important to control the FMD outbreaks in many countries. Thus, a lot of different approaches have been attempted. At the beginning, the killed or inactivated vaccines have been practiced. However, FMDV vaccines like other killed antigens do not induce broadly reactive long-term protection; require multiple vaccinations to

maintain good levels of herd immunity. Despite, conventional binary ethyleneimine inactivated vaccines emulsified with adjuvant have been widely used in Asia, Africa, and South America for effective control and eradication programs. Several novel approaches have been applied to develop alternative FMD vaccines, including construction of modified live-virus [11,12], biosynthetic proteins [13,14], synthetic peptides [15,16], naked DNA vectors [17,18], oral vaccine produced from transgenic plants [19,20] and recombinant viruses. Recombinant adenovirus [21-23], recombinant vaccinia virus [24], pseudorabies or fowlpox-vectored vaccine [25,26] and recombinant baculoviruses have been developed to express virus like particles (VLP) [27,28].

In the present study, we attempted to develop a novel strategy for FMDV vaccine using porcine reproductive and respiratory syndrome virus (PRRSV) replicon as a vector. Our results indicate that a PRRSV replicon vector expresses FMDV structural protein as well as N protein of PRRSV *in vitro*. The novel strategy will be useful to enhance the efficacy of FMDV vaccines in the future.

## Materials and Methods

### PRRSV vector construction

PRRSV infectious clone (PRRSVK418DM) was used for the genetic manipulation of PRRSV in this study. At the beginning, PRRSVK418DM was constructed as an expression vector, since then further modifications were carried out in the infectious clone. To introduce enzyme sites (*Ascl* and *Fsel*) and artificial transcriptional initiation sequences (TRS), the

**Table 1.** Primer nucleotide sequences and applications

Primer	Nucleotide sequences (5'-3') <sup>a)</sup>	Application
PK418F-11643	GCTATGCCTCGTACATCCGAGTT	PCR to separate ORF1b/2
PK418R-12193	GCCAACCGGGCATGGTGAAGC	PCR to separate ORF1b/2
12AF	<u>GGCGCGCCATTAAATGGCCGGCGTCCGTGGCAACCC</u> CTTAACCCAGAGTTTCAAGCGGAAG AATGAAATGG GGTCCATGCAAAGCCTCTTGAC	PCR to insert <i>Ascl</i> , <i>Swal</i> , <i>Fsel</i> , and TRS6
12AR	TCTTCCGCTGAAACTCTGGTAAAGGGGTTGCCACCGAACGGCCGGCCATTAAATGGCGCGCCTCAATTAG GCCTAAAGTTGGTCAATGACAGGGCC	PCR to insert <i>Ascl</i> , <i>Swal</i> , <i>Fsel</i> , and TRS6
PacI R	GGCTCTCAAGGGCATCGTAATTAACTGTT	PCR to N gene
PRS17F	GTGTCCATTGTTGATA <u>TCTATGCCAAATAACAACGGCAAG</u>	PCR to N gene, introduce <i>EcoRV</i>
MF	GAG CAA CAC CTT CTT GGC GGGACT TGC CCA GTA TTA TAC	Site directed mutagenesis
MR	GTA TAA TAC TGG GCA AGT CCC GCC AAG AAG GTG TTG CTC	Site directed mutagenesis
SPF	AAGGGCGGCCATGGGCGCCGGCCAATCCAG	PCR to insert <i>Ascl</i> site in P12A3C
SPR	<b>GTGGGCCGGCCTCATT</b> CATTAGGCGCG	PCR to insert <i>Fsel</i> site in P12A3C

PCR, polymerase chain reaction.

<sup>a)</sup>Restriction sites are underlined. TRS6 is mentioned in bold letters.

primers 12AF and 12AR (Table 1) were synthesized by Macrogen (Seoul, Korea). For construction of the PRRSV expression vector, the regions flanking the ORF1b/ORF2a junction were amplified as two fragments by polymerase chain reaction (PCR) using the primers PK418-11643F and 12AR, and 12AF and PK418-12193R, respectively. An overlap PCR was performed by primer set Pk418-11643F and Pk418-12193R using the mixture of purified two amplified fragments as a template, to get one completely modified fragment, which was ligated in pCR8/GW/TOPO using the TA cloning kit (Invitrogen, Carlsbad, CA, USA) and transformed into DH5 $\alpha$  cells. The recombinant clones were analyzed by recombinant plasmid isolation, restriction enzyme digestion and sequencing. The sequence confirmed recombinant plasmid was further allowed to prepare inserts to replace the modified junction fragment into PRRSVK418. The PRRSVK418DM and insert containing recombinant plasmid were digested by *Hpa*I and *Eco*RV restriction enzyme. The restriction enzyme digested PRRSVK418DM and modified inserts were purified using a Dokdo-Prep Gel Extraction Kit (ELPIS BIOTECH, Daejeon, Korea). The purified insert and linearized PRRSVK418-DM were ligated and transformed in to DH5 $\alpha$  competent cells. The PRRSV vector positive clone was analyzed by recombinant plasmid isolation, restriction enzyme digestion analysis and sequencing.

#### FMDVP12A3C gene construction

The P12A3C gene of FMDV type "O" was codon optimized to express in eukaryotic cells and synthesized by Bioneer Corp. (Daejeon, Korea). FMDV-type "O" isolate, Genbank accession no. HM229661 was used as a reference to design P12A3C gene. The synthesized gene was amplified by PCR using primers (SPF and SPR) to introduce two enzyme sites *Ascl* and *Fse*I on 5' and 3' region, respectively. In addition, site directed mutagenesis was carried out to modify internal *Fse*I site using the oligonucleotide primers without changing codon using primers MF and MR and site-directed mutagenesis kit (Qiagen, Valencia, CA, USA). The amplified full length P12A3C gene was cloned into pCR8/GW/TOPO TA cloning vector and the sequence was analyzed at Macrogen.

#### Construction of PRRSV replicon with FMDV

The P12A3C containing plasmid was digested using *Ascl*, *Fse*I, and *Xho*I restriction enzymes. PRRSV expression vector was digested by *Ascl* and *Fse*I; The prepared vector and inserts were ligated and transformed into DH5 $\alpha$  cells. The inserted

fragment was analyzed by gene sequencing. The P12A3C containing full length PRRSV was further allowed to modify as a replicon. To construct replicon along with P12A3C, The N gene was amplified using primers PRS17F and *Pac*I R. The amplified PCR product was ligated into a cloning vector pCR8/GW/TOPO using the TA cloning kit (Invitrogen); transformed into *Escherichia coli* DH5 $\alpha$  cells, and the sequences were analyzed using gene sequencing. FMDV gene containing PRRSVK418DM and the N gene containing plasmids were digested with *Eco*RV and *Pac*I enzymes, and allowed for ligation. The ligated fragments were transformed into DH5 $\alpha$  cells, followed by plasmids were isolated and sequenced.

#### In-vitro transcription

Replicon plasmids were isolated using a QIAfilter Plasmid Maxi Kit (Qiagen, Hilden, Germany) followed by identification by electrophoresis, restriction enzyme map identification. Ten micrograms of replicon plasmid was linearized by cleavage with the restriction enzyme either *AcII* or *Pac*I, and the linear DNA was further treated with mungbean nuclease followed by recovering DNA using phenol : chloroform extraction and purification. RNA was synthesized by using mMESSAGEMACHINE Kit (Ambion, Austin, TX, USA) and purified linearized-plasmid as template, according to the manufacturer's protocol, and including treatment of the RNA with DNase to remove input plasmid. The RNA was dissolved in nuclease-free water.

#### Cells and replicon transfection

MARC-145 cells (African green monkey kidney epithelial cell line) were used for electroporation mediated transfection. MARC-145 cells were grown and maintained in Dulbecco's modified Eagle's low glucose medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO<sub>2</sub>. Cells were transfected with replicon transcribed RNA by electroporation. MARC-145 cells were seeded into a 6-well plate and grown for 2 days, to reach the log phase before transfection. The cells were washed once with DMEM with 5% FBS, and twice with DMEM containing 1.25% dimethyl sulfoxide (DMSO) without FBS. An approximately, 2 × 10<sup>6</sup> cells in 400 μL of phosphate buffered saline (PBS) were electroporated with approximately 10 μg of *in vitro* transcripts along with 10 μg of total RNA isolated from MARC-145 cells by pulsing once using Bio-Rad Gene PulserXcell (Bio-Rad, Hercules, CA, USA) at 250 V, 950 μF in a 4.0 mm cuvette. The electroporated cells were transferred into a DMEM containing 10% FBS and 1.25%

DMSO in a 60 mm cell culture plate for virus recovery and in a separate plate for immunofluorescence staining; incubated at 37°C under 5% CO<sub>2</sub>. The 16 hour postelectroporated cells were washed and the medium was replaced with DMEM containing 5% FBS. The supernatant from the 96 hour post-electroporated cells in 60 mm plate was collected, clarified, and used for further studies.

### Immunofluorescence antibody assay

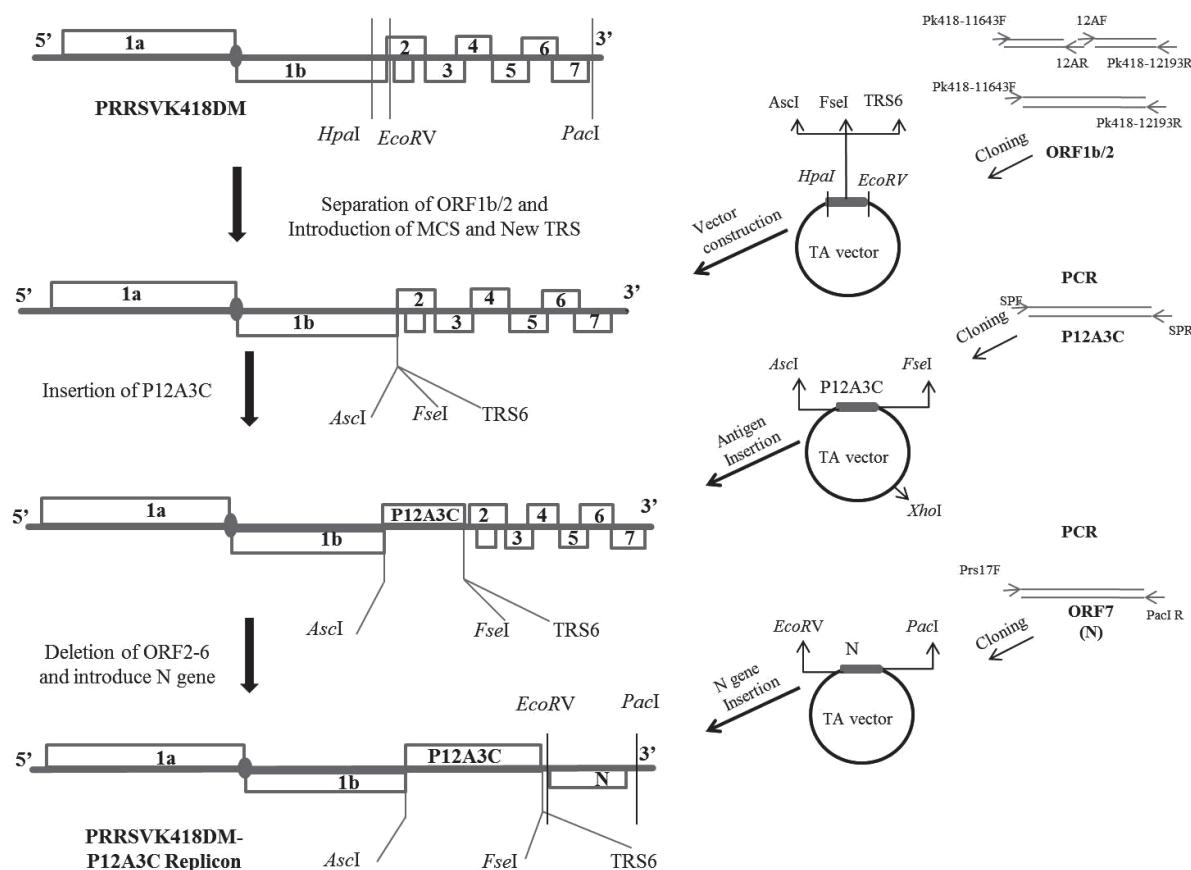
MARC-145 cells were grown overnight to 70% confluence on 48 well plates. Cells were infected with 100 µL of replicon alone or co-infected with PRRSV at a multiplicity of infection of 0.1 for 48 hours at 37°C. In addition, a separate well containing 96 hour post-transfected cells were infected with the same concentration of PRRSV. At 48 hour postinfection, cells were washed once with PBS and fixed with cold methanol : acetone (1:1) for 10 minutes at room temperature, followed by the cells

air dried for 5 minutes and washed once with PBS. Cells were incubated with PRRSV specific MAb SDOW-17 and FMDV type "O" VP1 MAb in PBS for 1 hour at 37°C. After washing 3 times in PBS, cells were incubated for 30 minutes with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (H + L) secondary antibody. The cells were washed 3 times in PBS and fluorescence was observed under the fluorescent microscope.

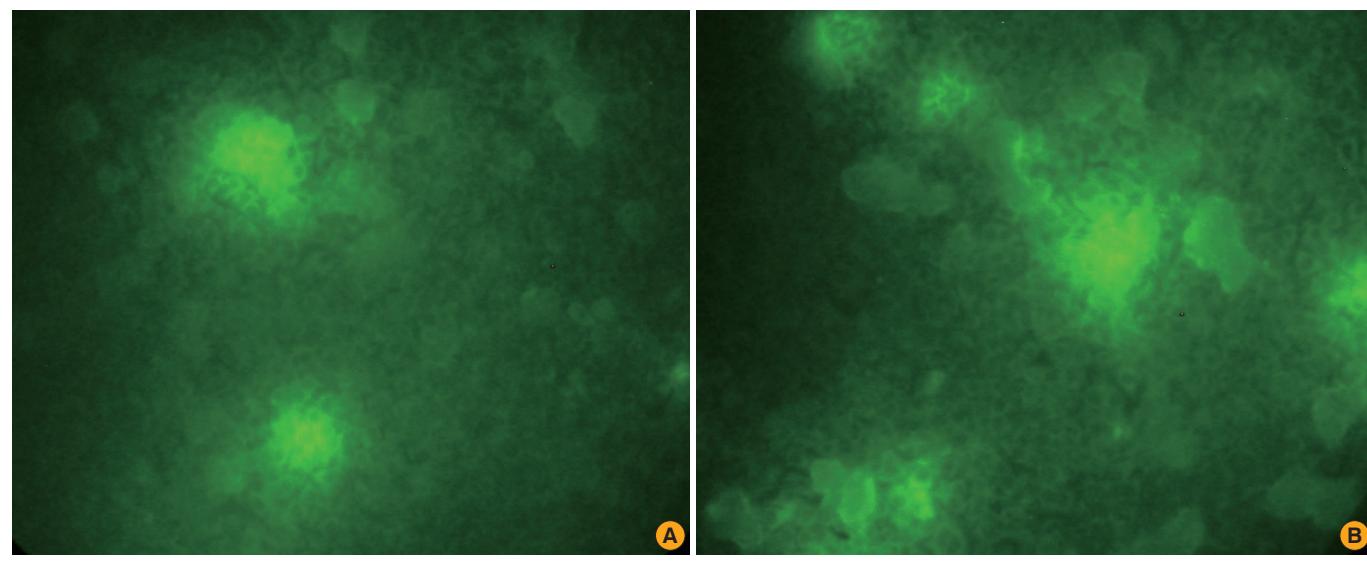
## Results

### Construction of PRRSV replicon with FMDV

The construct of PRRSV replicon containing FMDVP12A3C was successfully achieved by following three strategies: Construction of PRRSVK418DM vector, Insertion of P12A3C between ORF1b/2 of PRRSVK418DM, and the deletion of ORF's 2 to 6 of PRRSVK418DM. The PRRSVK18DM infectious clone



**Fig. 1.** Schematic representation of the PRRSVK418DM-P12A3C replicon construction strategy. Porcine reproductive and respiratory syndrome virus (PRRSV) replicon containing FMDVP12A3C was constructed by three strategies: PRRSVK418DM vector construction, P12A3C insertion between ORF1b/2 of PRRSVK418DM, and the deletion of ORFs 2 to 6 of PRRSVK418DM. All the modification was performed by overlap polymerase chain reaction and direct cloning procedure. The used overlapping primers and flanking primers are mentioned. The italic letters represent the restriction enzymes used for reverse genetic manipulation. PCR, polymerase chain reaction; TRS, transcriptional initiation sequences.



**Fig. 2.** Analysis of foot-and-mouth disease virus (FMDV) and porcine reproductive and respiratory syndrome virus (PRRSV) gene expression without helper virus by immunofluorescence antibody assay. MARC-145 cells were infected with the supernatant from the replicon. At 48 hour postinfection, the cells were processed for indirect immunofluorescence detection using antibodies specific for PRRSV N protein (anti-PRRSV N Ab) (A), FMDV-VP1 (anti-VP1 MAb) (B).

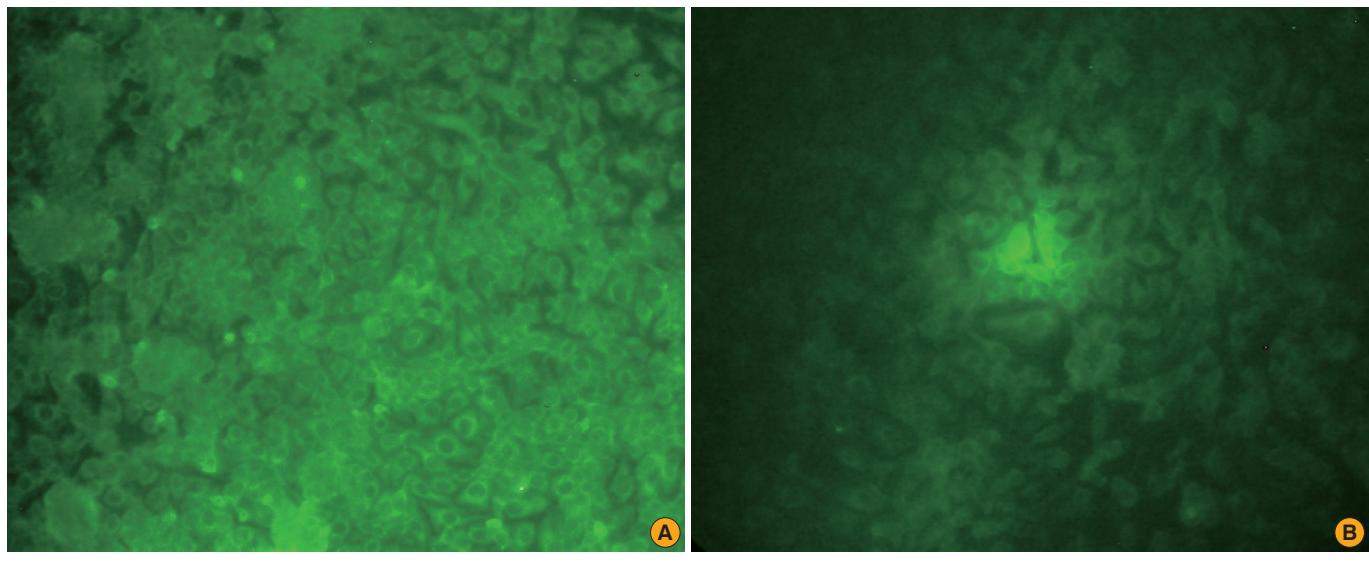
was used for this complete study, and the schematic representation of the PRRSV replicon containing P12A3C construction was mentioned in Fig. 1. PRRSVK418DM is about 19 kbp in size and the complete sequences and restriction enzyme map was obtained. Based on the restriction enzyme sites, we chose enzyme sites *Hpa*I and *Eco*RV, located at nucleotide position 11671 and 12163, respectively. The region between these sites was about 490 bp, and we designed primer set to flank about 570 bp from ORFs 1b and 2. The ORF's 1b and 2 are non-overlapping and only one nucleotide persists between ORF1 and 2. Moreover, the TRS2 is located on ORF1b and positioned 26 nucleotides upstream from the start of ORF2. The ORF1b/2 junction was modified by introducing multiple cloning sites (*Ascl-Swa*I-*Fse*I), followed by additional TRS6, which is located from 17 nucleotides downstream of *Fse*I site. The modified junction was ligated back to PRRSVK18DM to enable complete expression vector. Thus, any foreign gene can be inserted using enzymes *Ascl* and *Fse*I.

The gene coding P12A3C was inserted between ORF1b and 2. Thus, TRS2 drive the transcription of P12A3C and the downstream of inserting genes TRS6 will drive the transcription of ORF2 without any interruption. To construct a replicon, the clone was further allowed to remove ORFs 2 to 6 of PRRSVK418. To remove ORFs 2 to 6, the enzyme *Eco*RV and *Pac*I, located downstream of 3' UTR region, was chosen. The size between ORFs 2 and 6 was about 2.8 kbp. Therefore, we

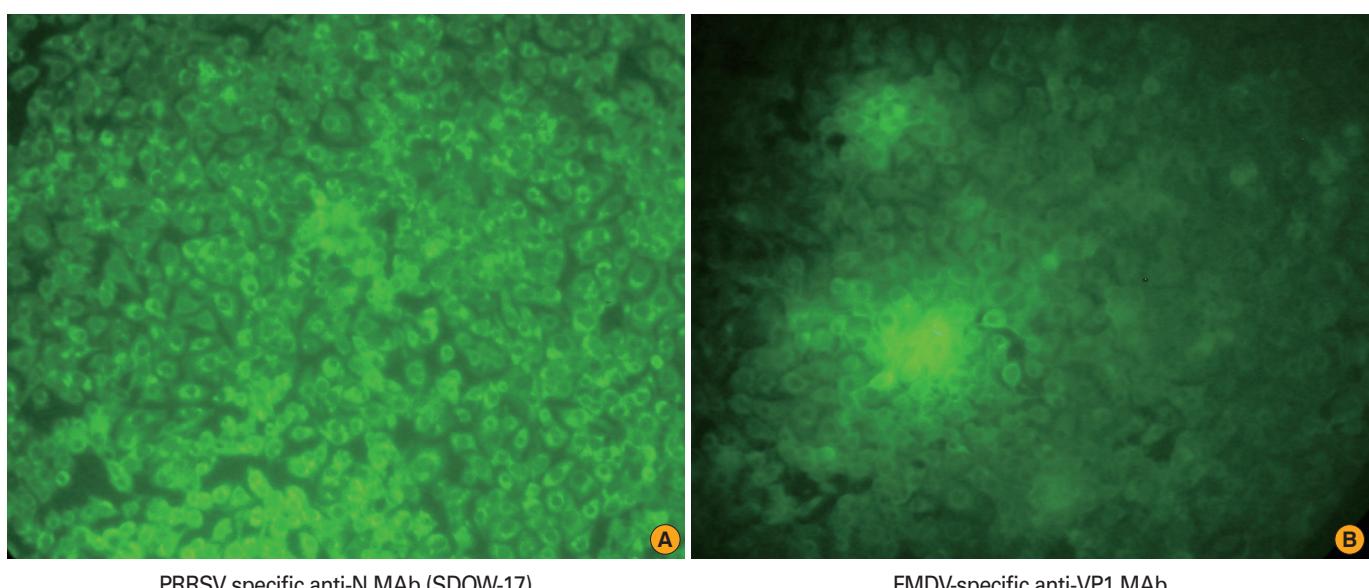
have carefully designed a primer containing *Eco*RV site and primers (PRS17F) flanking *Pac*I regions (PacIR). Eventually, the PRRSV N gene was cut with restriction enzymes (*Eco*RV and *Pac*I) and replaced into the backbone of PRRSVK418DM. The restriction enzyme digestion and sequence analysis results was confirmed the manipulation of replicon vector systems. The enzyme *Eco*RV was located an approximately 90 nucleotides downstream from the starting point of ORF2. Thus, the N gene is added 90 bp an extra from the upstream of the N gene start point. However, the sequence results revealed that it does not affect the expression of N gene. In addition, the orientation of the gene was analyzed by ORF findings, which proved that the sequences were manipulated without changing the amino acids and orientation of ORFs. The manipulated PRRSVK418DM containing P12A3C of FMDV is mentioned as PRRSVK418DM-P12A3C replicon. In the case of replicon, TRS6, which is located at 22 nucleotides downstream of the P12A3C, will drive the transcription of ORF7 or N gene. The PRRSVK418DM-P12A3C replicon was thought to be the only gene replicative due to the lack of PRRSV structural genes and not possible to form a complete PRRS virus particle.

#### Expression of FMDV along with PRRSV N protein

The expression of P12A3C and N gene of PRRSV was confirmed by immunofluorescence antibody assay (Fig. 2). The



**Fig. 3.** Identification of the P12A3C expression with helper virus by immunofluorescence antibody assay. MARC-145 cells were co-infected with the supernatant from the replicon and PRRSVK418DM. At 48 hour postinfection, the cells were processed for indirect immunofluorescence detection using antibodies specific for porcine reproductive and respiratory syndrome virus (PRRSV) N protein (anti-PRRSV N Ab) (A), foot-and-mouth disease virus (FMDV)-VP1 (anti-VP1 (MAb) (B).



**Fig. 4.** An alternative analysis of the P12A3C expression with helper virus by immunofluorescence antibody assay. The replicon transfected MARC-145 cells were directly infected by PRRSVK418DM at 96 hour post-transfection. At 48 hour postinfection, the cells were processed for indirect immunofluorescence detection using antibodies specific for porcine reproductive and respiratory syndrome virus (PRRSV) N protein (anti-PRRSV N Ab), foot-and-mouth disease virus (FMDV)-VP1 (anti-VP1 MAb).

supernatant from the 96 hour post-transfected cells were collected and infected with fresh MARC-145 cells. The cells were indirect-immunostained with a monoclonal antibody against the N protein of PRRSV (SDOW17) and against VP1 protein of FMDV (anti-VP1 MAb) at 48 hour postinfection. The result

shows fluorescence on both VP1 and N proteins and it is confirmed the expression of both P12A3C protein and N genes. However, the expression was unclear, because of the replicon particle (RP) does not spread effectively other cells. To confirm the expression of N gene and P12A3C, We did one step

reverse transcription polymerase chain reaction using specific primers for N gene and VP1 gene (data not shown). Furthermore, the cytopathic effect (CPE) was not observed. The immunofluorescence antibody assay (IFA) and CPE observation results revealed that the FMDV structural gene continuously expressing in PRRSV replicon but not produce infectious particles. On the other hand, the inability of spreading RP or infection of the replicon alone was not able to effectively infect neighboring cells, without PRRSV structural protein. Therefore, the PRRSVK418DM virus was used either to co-infected with replicon particles or the transfected cells were infected with PRRSVK418DM, which may help to spread into neighbor cells, thus allow the clear picture of P12A3C expression. The co-infected cells were analyzed after 48 hour infection by IFA using anti-N protein and anti VP1 MAb. The IFA results indicate that the inserted P12A3C express well and spread to other cells with the help of helper virus (Figs. 3, 4). Thus, the IFA pictures confirmed that the replicon is continually expressing P12A3C as well as N protein of PRRSV in *in vitro*, and possible to spread the cells with the help of the helper PRRSV.

## Discussion

FMD, as a global animal disease, affects millions of animals worldwide. In South Korea, about 3,700 farms had been seriously affected due to the serotype O, during November 2010 to April 2011; it was controlled by implementing the emergency vaccination program through the supplement of O1 Manisa vaccines [10,29]. However, still unresolved problems remain to develop effective alternative vaccines. Despite, inactivated vaccines have been widely used to control FMD; there are some drawbacks in inducing long term protection and safety in production. Therefore, the development of new alternative vaccine is essential for an effective control of FMD [1]. To overcome the limitation of inactivated vaccines, a number of novel vaccine strategies have been applied. On the other hand, the concern about escaping viruses from the production plant and the reversion of inactivated virus are forced to develop novel vaccines with safety.

To concern about the safety, it was thought that the sub-unit vaccines or synthetic peptides might be an alternative for vaccine development. The studies have been reported that the VP1 gene was responsible for the induction of protective neutralizing antibodies [1,30]. Nevertheless, the later studies have proved that the VP1 could show only the partial

protection. Thus, structural characterization has been played important role for the development of complete protection and few studies have been carried out the effect of protection using the complete P1 polyprotein [30,31]. Eventually, the later studies reported that the NS protein 3C plays crucial role in P1 processing and assembly. Subsequent studies, suggest that 3C proteinase (3C<sup>pro</sup>) is essential for the processing of capsid precursor, which facilitates to induce neutralizing antibodies and a protective immune response. The FMDVP1 along with 3C<sup>pro</sup> could induce both humeral and cell mediated immunity [23,32]. Furthermore, the P1 region along with 2A and 3C have also been shown strong protection [21]. Therefore, to concern about the importance of 3C<sup>pro</sup> and 2A peptide, we designed an antigen P12A3C from serotype O. The P12A3C antigen expression was confirmed by our previous studies (unpublished data). The complete P12A3C is about 3.4 kbp and this poly protein could be expressed as a single protein and the 3C<sup>pro</sup> has the ability to auto processing of P1, as a result it can produce complete VLP. However, the large size of P12A3C was not able to accept by all the vector systems. We have tried with our previous studies that the development PRRSV vector (unpublished data), which could express FMDV antigen, but it was unstable to maintain their large size genome inserts, when allowed for multiple passages. However, the large genome size vectors such as adenovirus vector or baculovirus vector systems could be possible for express large genome size antigen and could produce VLPs [5,33]. We developed a strategy to express P12A3C in PRRSV vector, which has the limited host specificity and advantages to deliver vaccines for swine. The replicon vector can continuously replicate in the transfected cells in the presence of essential region comprising in the ORF7 of PRRSV, and the lack of major structural protein of PRRSV. Moreover, the replicon vector system will be more promising with the safety rather than the commercial inactivated vaccines. Furthermore, the replicon virus could be impossible to revert as an emerging complete virus without complete structural protein. Therefore, the replicon vector system will be one of the safest and alternatives for commercial vaccine development in the future. However, further detailed studies are still required to develop as a complete vaccine and to improve the efficacy of vaccines.

Replication defective, recombinant vector vaccines are one of the key interests in the alternative vaccine development. For instance, Adenovirus and pox virus have been widely used for the efficient expression of heterologous genes. Adenovirus

5 has the ability to carry about 5-8 kb of foreign genes, which facilitate the production of complete FMDV structural proteins [34]. Apart from the adenovirus vector systems, other vector systems have also been developed such as recombinant vaccinia virus, fowlpox virus and pseudorabies virus [34]. To control swine viruses, researchers have been attempted PRRSV as recombinant vector vaccines. PRRSV is an enveloped, single-stranded positive-sense RNA virus, a member of the order *Nidovirales*, family *Arteriviridae*. Moreover, PRRSV is about 15 kb size of RNA virus, thus allows the easier handling and manipulation of their genomes. The natural genetic mutation or deletion of NS protein region 2 (NS2) has been found in the field strain of PRRSV [35]. The natural deletions enabled the possibility of insertion of foreign genes or marker genes in the nonessential region of PRRSV NS2. In order to develop replication competitive PRRSV recombinant vectors, marker genes such as green fluorescent protein (GFP), enhanced GFP (EGFP) and luciferase have been successfully inserted to the nonessential region of PRRSV NS2. In recent, NP gene fragment of Newcastle disease virus has been inserted successfully into the deletion site by reverse genetic manipulation, and found stable expression [36]. Nevertheless, some of the results indicate that the manipulated viruses in NS2 region don't depict the consistency of foreign gene expression after several passages.

The separation of PRRSV overlapping ORFs has not been interrupted the sub genomic RNA synthesis and the virus replication [37]. The direct insertion of PCV2 genes in the region between ORF1b and 2 failed to express the inserted gene due to the lack of TRS and interruption on subgenomic RNA synthesis. Subsequently, a novel approach attempted by Pei et al. [38], by introducing artificial TRS6, downstream of inserting foreign genes, enable the stability of inserting genes more than 30 passages, proved the immune response for both PRRSV and inserted foreign gene. Therefore, we chose PRRSV as a vector to insert P12A3C gene between ORF1b and ORF2 and an addition of artificial TRS6, introduced downstream of P12A3C. Before, developing PRRSV replicon vector, we confirmed our vector system the expression of EGFP, P12A3C and other foreign gene expression from our previous study (unpublished data). The large size of P12A3C genome accommodation in PRRSV, and the advantages of replicon vector, we tried PRRSV replicon vector to express P12A3C.

Many replicon based vaccines have been under the development for protecting many diseases including medicine and veterinary medicine. Replicon vectors are derived from either

positive- or negative-strand RNA viruses, and applied to vaccines and gene therapy. The replicon vector system has a lot of advantages such as safety, high level expression of heterologous genes, only cytosolic expression of recombinant proteins, unable to spread neighbor cells due to the lack of structural proteins, easy manipulation by reverse genetic systems, multivalent vaccine development and unable to revert to virulence. Furthermore, replicon based vector vaccines possibly can stimulate both the humoral and the cellular mediated immune system [39]. Therefore, we chose the replicon based PRRSV vector system to express FMDVP12A3C protein. In our PRRSV replicon system, we have constructed without PRRSV ORFs 2, 3, 4, 5, and 6, and replaced FMDVP12A3C, which is more or less equal to the size of PRRSV structural protein. Thus, PRRSV replicon can access a large genome size of antigen without any trouble. Our replicon vector is similar like the previous report without ORFs 2 to 6, and the previous reports analyzed heterologous gene expression in both *in vitro* and *in vivo* using GFP marker genes [40]. Our results revealed that replicon vector systems confirmed expression of P12A3C *in vitro*, and we will carry out *in vivo* studies in future. We believe that the *in vivo* studies will also produce similar result like previous replicon vector systems [40]. Moreover, the obtained results revealed that the constructed PRRSV replicon express the FMDV antigen in *in vitro* and the replicon vector strategies are promising for an effective development of novel vaccines.

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